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Competition for Formation of Nucleosomes on Fragmented SV40 DNA: A Hyperstable Nucleosome Forms on the Termination Region†

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ABSTRACT: We have studied the relative abilities of different simian virus 40 (SV40) DNA segments to reconstitute into nucleosomes in vitro. The SV40 genome was separated into 15 discrete fragments by restriction endonuclease digestion and reconstituted with calf thymus core histones under conditions of varying histone-to-DNA ratios. Three fragments show very different abilities to form nucleosomes when low histone-to-DNA ratios require all fragments to compete for available histones. Two of these fragments, both from within protein-coding regions, are significantly underreconstituted. The third fragment, covering the SV40 termination region, competes much more effectively for histones than the other 14 fragments. The fragment containing the SV40 origin region formed nucleosomes with about average probability. Overall, the SV40 fragments differed by approximately an order of magnitude in their abilities to support nucleosome formation in vitro. The stability of the nucleosomes was measured by challenge with high concentrations of the destabilizing reagent heparin. The fragment that reconstituted most effectively also formed nucleosomes that were unusually stable to heparin challenge. These observations are intriguing since this fragment contains the sequences where replication of SV40 DNA commonly terminates and where early messenger RNA synthesis may terminate as well. The existence of unique hyperstable nucleosomes in this region suggests the interesting possibility that such nucleosomes may assist in termination events by assisting in the pausing of replication or transcription complexes.

It is generally accepted that chromatin structure may modulate processes that are basic to gene expression, such as transcription and replication. However, there is still controversy over the question of how chromatin structure is controlled or maintained. A list of parameters that may affect the structure of chromatin would include DNA sequence, chemical modifications of DNA and histones, the presence of nonhistone proteins and histone variants, and the state of torsional stress of the DNA. An understanding of the relative importance of these parameters to the maintenance of a specific chromatin configuration in vivo would enhance our understanding of how gene expression is regulated.

The chromatin structure of the simian papovavirus virus, SV40,[1] has been studied extensively. Late after infection, replicated SV40 DNA associates with host cellular histones to form a repeating structure of nucleosomes. This viral nucleoprotein complex is called the minichromosome and has served as a convenient model for the chromatin of higher eukaryotes. Several studies have shown that in a subpopulation of SV40 minichromosomes nucleosome placement is nonrandom with respect to DNA sequence (Persico-DiLauro et al., 1977; Nedospasov & Georgiev, 1980; Cereghini et al., 1982). In these complexes, a nucleosome-free region of approximately 300–400 base pairs exists at map positions 0.66–0.75 (Varshavsky et al., 1978, 1979; Scott & Wigmore, 1978; Waldeck et al., 1978; Saragosti et al., 1980; Jacobovits et al., 1980; Beard et al., 1981; Robinson & Hallick, 1982). Since this gap is positioned over the origin of the replication, the binding sites for the viral regulatory protein, T antigen, and the promoters for early and late SV40 mRNAs, it is presumed that this structure is relevant to the processes of DNA replication and transcription.

The parameters responsible for establishing the nucleosome-free gap in SV40 chromatin are not fully understood.

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[1] Abbreviations: bp, base pair(s); EDTA, (ethylenedinitrilo)tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SV40, simian virus 40; Tris, tris(hydroxymethyl)aminomethane.

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The basic question of whether DNA sequence alone may affect nucleosome placement on SV40 is still controversial. Three studies have been reported that attempted to address this question directly by reconstituting minichromosomes from purified SV40 DNA and core histones (Wasylyk et al., 1979; Simpson & Stein, 1980; Hiwasa et al., 1981). The positions of the nucleosomes on the DNA were assayed by the accessibility of specific DNA sequences to restriction enzymes or by electron micrograph mapping. The results of two of these studies suggested that the origin-containing sequences did not form nucleosomes as readily as other sequences of SV40; however, the third study found no evidence of a nucleosome-free region at the origin.

Since the frequency of digestion by single-site restriction enzymes may not be a sensitive probe for nucleosome placement over a 300–400-bp region, we decided to develop an assay that would allow for a simple, yet accurate, quantitation of the results. Rather than reconstituting whole SV40 DNA and then asking which sequences preferentially bound histones, we chose to reconstitute a mixture of restriction fragments representing the entire SV40 genome. Since these restriction fragments could be readily separated from each other, we were able to analyze the mixture of reconstituted fragments for the content of each individual fragment directly.

Our results indicate that the DNA fragment containing the sequences determined to be nucleosome-free in minichromosomes reconstitutes normally when compared to other fragments of the SV40 genome. However, three other fragments demonstrate sequence-specific differences in their ability to reconstitute. Two of these fragments do not reconstitute as well as the majority of the SV40 fragments. A third fragment reconstitutes more effectively than the average and, in addition, forms exceptionally stable associations with histone octamers. This novel observation is intriguing since this fragment corresponds to sequences that are diametrically opposed to the origin region of SV40 and that may encompass the termination sites for DNA replication and early mRNA transcription.

Materials and Methods

Preparation of RNA. Form I SV40 DNA was prepared from CV-1 cells infected with SV40 virus, strain 776. The DNA was cleaved with the restriction endonucleases *Hin*dIII, *Kpn*I, and *Mbo*I according to the manufacturer's instructions (Bethesda Research Laboratories). The mixture of restricted fragments was treated with bacterial alkaline phosphatase (Worthington) for 1 h at 65 °C in 50 mM Tris, pH 8.0, and 2 mM $MgCl_2$. EDTA was then added to 10 mM, and the reaction mixture was incubated for an additional 15 min at 65 °C to inactivate the phosphatase. The reaction was extracted 3 times with phenol and then 2 times with ether. The DNA was precipitated at −20 °C by addition of sodium acetate to 0.3 M and 2.5 volumes of ethanol. A total of 1 μg of dephosphorylated SV40 restriction fragments was labeled at the 5′ end with [γ-^{32}P]ATP and polynucleotide kinase (P-L Biochemicals), as described by Maxam and Gilbert (1980). The labeled fragments were stored in 10 mM Tris, pH 8.0, and 1 mM EDTA, at −20 °C.

The DNA used as carrier during the reconstitution was isolated by three phenol extractions and two chloroform–isoamyl alcohol extractions of nucleosomal chromatin that was generated by micrococcal nuclease digestion of calf thymus nuclei. This DNA was stored in 10 mM Tris, pH 8.0, and 1 mM EDTA, at a concentration of 2.0 mg/mL.

Isolation of Core Histones. Histones were isolated by 0.4 N H_2SO_4 extraction of calf thymus nuclei suspended in distilled water. After acetone precipitation, the histones were resuspended in distilled water and extracted 3 times with 5% perchloric acid to remove H1. The core histones were redissolved in distilled water to a concentration of 7.2 mg/mL and stored at −70 °C in small aliquots. The absence of H1 and the general purity of this preparation were confirmed on an 18% polyacrylamide/0.1% sodium dodecyl sulfate gel (Bonner & Pollard, 1975).

Reconstitution of Nucleosomes. Reconstitution was accomplished with the rapid-mixing procedure described by Chao et al. (1979), with the following modifications: 245 μg of calf thymus core histones was incubated with 7.2 M urea and 20 mM HCl in a total volume of 100 μL during the first step of the histone denaturation procedure. The pH of the histone mix was brought to 7.5, and after dilution to a histone concentration of 0.5 mg/mL and a urea concentration of 1.5 M, 60 μL of this mix was added slowly to an equal volume of DNA mix. The DNA mix contained 2×10^6 cpm of ^{32}P-end-labeled SV40 restriction fragments and 30 μg of calf thymus carrier DNA in 2 M NaCl and 10 mM Tris, pH 7.5. The mixture of histones and DNA was incubated at 37 °C and diluted to a final volume of 200 μL over a period of 4 h by four stepwise additions of 20 μL of water. This resulted in a final concentration of 0.6 M NaCl, 0.45 M urea, and 0.15 mg/mL DNA.

Normally the reconstitution was performed at a histone-to-DNA weight ratio of 1, so that the final concentration of histones in the reconstitution mix was 0.15 mg/mL. In experiments where different histone-to-DNA weight ratios were used during reconstitution, the DNA concentration was held constant while the amount of histone added to the initial denaturation buffer was varied over a 5-fold range. Mock reconstitution with BSA were carried out by substituting an equivalent amount of BSA for histone proteins in the initial denaturation buffer.

Heparin challenge experiments were performed by reconstitution at a histone-to-DNA ratio of 1, as described above. After reconstitution was complete, the mixture was diluted to a NaCl concentration of 0.45 M, and heparin was added to a final concentration of either 0.226 or 1.13 mg/mL. The reconstitution mixture was incubated at 37 °C in the presence of heparin for an additional hour and then sedimented on a sucrose gradient.

Fractionation of Reconstitutes. The 200-μL reconstitution mix was sedimented in a 5–20% sucrose gradient containing 10 mM Tris, pH 8.0, 1 mM EDTA, 1 mM PMSF, and 50 mM NaCl. Sedimentation was at 20 °C for 4 h at 58 000 rpm in an SW60 rotor. Five-drop fractions were collected from the bottom and monitored for ^{32}P-labeled DNA by Cerenkov counting. Fractions corresponding to mononucleosomes, dinucleosomes, and higher order nucleosomes were pooled separately. The DNA in each set of pooled fractions was deproteinized by incubation with 0.1% sodium dodecyl sulfate, followed by two phenol extractions and one chloroform extraction. The DNA was subsequently concentrated by two ethanol precipitations and the DNA pellet resuspended in 30 μL of a loading mix consisting of 5% glycerol, 90 mM Tris–borate, 2.5 mM EDTA, and 0.025% xylene cyanol and bromophenol blue. A total of 10 μL of each sample was applied to a 20- by 40-cm 0.6 mm thick 8% polyacrylamide gel cast in TBE buffer (Maniatis et al., 1975). Electrophoresis was for approximately 8 h at 2 W (constant power), until the xylene cyanol tracking dye had run 17 cm into the gel. The polyacrylamide gels were dried on a piece of Whatman 3MM paper and exposed at room temperature to Cronex 4 X-ray film (Du Pont) for varying amounts of time.

For gels such as that shown in Figure 2, each gradient fraction was ethanol-precipitated with the addition of NH_4OAc to 1 M and 2.5 volumes of ethanol. Each sample was resuspended in the dye mix (described above) to which 0.1% SDS had been added and applied to an 8% polyacrylamide/TBE gel containing 10% glycerol and 0.1% SDS. The gel was electrophoresed at 5 W for approximately 12 h, until the xylene cyanol tracking dye had reached the bottom of the gel. Autoradiographs were obtained by exposure of the gel to X-ray film at −70 °C.

In order to confirm the order of migration of the SV40 DNA fragments on polyacrylamide gels, the mixture of fragments was subjected to restriction endonuclease digestion prior to gel electrophoresis. The resulting restriction pattern was then used to map the relative positions of the DNA fragments on the gel (data not shown). This analysis indicated that two sets of fragments (the 237 and 240 bp and the 430 and 447 bp) run anomalously on these gels, since their relative positions are reversed in terms of the pattern expected on the basis of their lengths. The 240-bp fragment arising from the *Hin*dIII end at nucleotide 3477 and the *Mbo*I end at nucleotide 3715 was cut by the restriction endonuclease *Pvu*II but not by *Hpa*I. Likewise, the 237-bp fragment arising from the *Mbo*I end at nucleotide 2534 and the *Mbo*I end at nucleotide 2770 was cut by *Hpa*I but not by *Pvu*II. This is the expected result, confirming the identity of the fragments, since there is a *Pvu*II restriction site at position 3506, placing it on the 240-bp fragment, and a *Hpa*I restriction site at position 2666, which maps to the 237-bp fragment. In a similar manner, the identify of the 430- and 447-bp fragments was confirmed by restriction endonuclease digestions with *Eco*RI and *Hph*I.

Quantitation of Reconstitution. Each lane of the autoradiograph was scanned with an Optronics drumscanner (Optronics International Inc., System P-1000) in conjunction with a VAX 11/780 computer. A 5 mm wide wedge centered about the middle of each lane was scanned in increments of 100 μm^2. This measurement was repeated for a total of 3 times, and an average summation of the optical density along the length of each lane was calculated by computer program (GELSCAN). The output of this program was then plotted in the form of a graph of optical density vs. distance from the top of each lane.

A second set of computer programs (AUTINT and GELINT) was used to calculate the total optical density in each DNA fragment. This was accomplished by integrating the optical densities of all of the 100-μm^2 units associated with an intensity peak and subtracting the background intensity. This background was calculated by selecting small boxes of 2–3 mm in width, in areas corresponding to clear film on the autoradiograph, and averaging their intensities. The standard deviation of this background intensity was used to set a threshold limit for the identification of peaks. (Normally, a threshold of 5σ, where σ represents the standard deviation in the background intensities, was used.) Alternatively, the peak boundaries could be selected by the user.

In order to determine the range of integrated optical densities that was linear with respect to amount of radioactivity, a curve of optical density vs. time of exposure was drawn. Times of exposure that resulted in a linear film response were used to generate three to four different exposures from a single gel. The multiple exposures were scanned, and the log of the integrated optical densities of each DNA fragment was divided by the length of time of the exposure. These normalized values were averaged together to obtain a final value for the integrated optical density. The error in this mean value for optical density, which arose from scanning several time exposures of the same gel, could then be calculated as a sample variance.

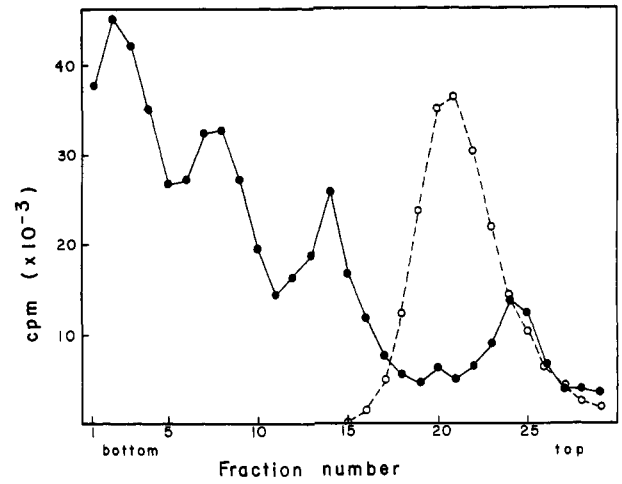

FIGURE 1: Sucrose gradient analysis of reconstituted SV40 DNA fragments. ^{32}P end-labeled SV40 restriction fragments were reconstituted at a histone-to-DNA (w/w) ratio of 1.0. Reconstitutes were run on 5–20% sucrose gradients in 10 mM Tris, pH 8.0, 1 mM EDTA, 50 mM NaCl, and 1 mM PMSF and analyzed as described under Materials and Methods. The dashed line corresponds to the position of mock-reconstituted SV40 restriction fragments run on a parallel sucrose gradient. These fragments were reconstituted with BSA in place of core histones and should thus correspond to unreconstituted DNA.

RESULTS

The restriction fragments used in the reconstitution experiments to be described were generated by a combination of *Kpn*I, *Hin*dIII, and *Mbo*I digestions of whole SV40 DNA. This combination of digestions produced 15 fragments ranging in size from 60 to 705 bp. We felt that this size range was appropriate since it allowed the separation of most of the fragments on polyacrylamide gels. In addition, the majority of the fragments fell into size clases that have been observed in mononucleosomes (100–190 bp), dinucleosomes (230–380 bp), trinucleosomes (380–540 bp), or tetranucleosomes (520–720 bp) isolated from Chinese hamster cells (Rall et al., 1977). Finally, the origin-containing fragment of 366 bp represents the median length in this distribution and should thus not be subject to any artifacts of reconstitution that may result from either short or long extremes of length.

The protocol for in vitro reconstitution of the SV40 restriction fragments with calf thymus core histones is similar to the one used previously in this laboratory (Chao et al., 1979). In addition to its demonstrated ability to produce specific nucleosome placement in vitro, this protocol has been shown to yield core particles resembling those isolated from nuclei (Chao, 1980). The reconstituted DNA fragments can be separated from unreconstituted fragments on isokinetic sucrose gradients. This technique offers the added advantage that it allows the separation of the different classes of nucleosomes: that is, mononucleosomes, dinucleosomes, trinucleosomes, and tetranucleosomes (Rall et al., 1977).

Figure 1 shows the sucrose gradient profile of a mixture of ^{32}P-labeled SV40 restriction fragments that were reconstituted in vitro with core histones. The majority of the DNA fragments are shifted toward the bottom of the gradient and appear in three distinct peaks relative to unreconstituted DNA. These peaks correspond to mononucleosome reconstitutes, dinucleosome reconstitutes, and "multinucleosome" reconstitutes, which, in our gradients, may consist of trinucleosomes as well as higher order nucleosomes. In general, we found no evidence

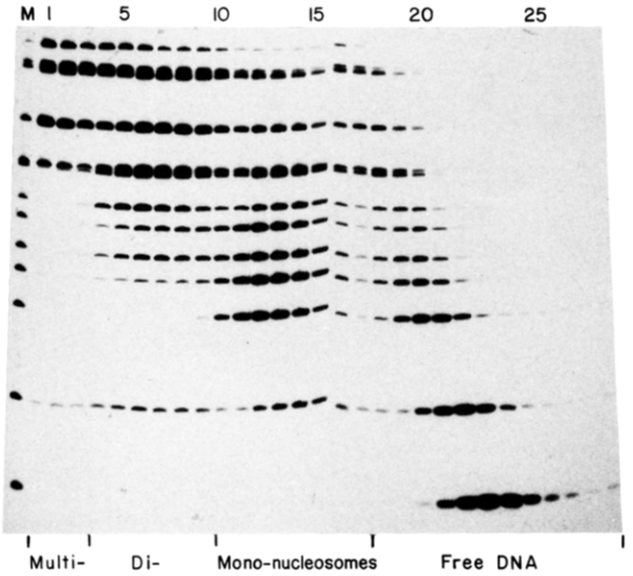

FIGURE 2: Autoradiograph of sucrose gradient samples of reconstitutes at a histone-to-DNA ratio of 0.6. Each gradient fraction was ethanol-precipiated with 1 M NH_4OAc and 2.5 volumes of EtOH. The entire sample was then applied to an 8% polyacrylamide gel containing 10% glycerol and 0.1% SDS. Electrophoresis was for approximately 12 h at 5 W, until the XC tracking dye had reached the bottom of the gel. The numbers at the top of the figure correspond to the gradient fraction numbers. M is a sample of the mixture of unreconstituted SV40 fragments. The vertical lines at the bottom indicate the limits of the gradient fractions contained within the multinucleosome, dinucleosome, mononucleosome, and free DNA peaks.

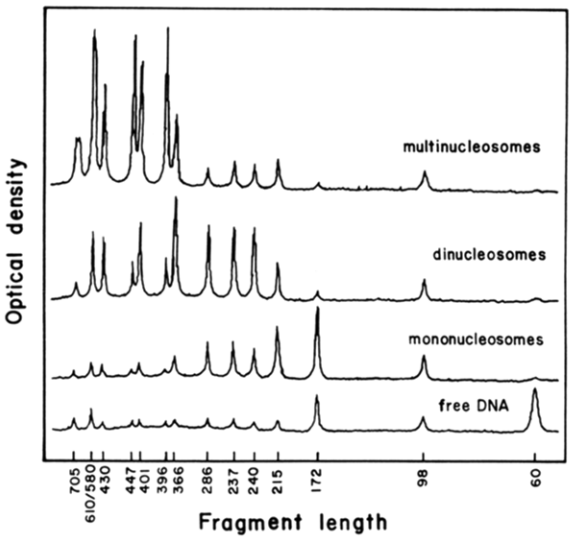

FIGURE 3: Densitometer tracings of polyacylamide gels. SV40 DNA fragments were reconstituted at a histone-to-DNA ratio of 1.0 prior to fractionation on sucrose gradients. Appropriate gradient fractions were pooled and deproteinized by phenol/chloroform extraction. The pooled samples were applied to four lanes of an 8% polyacrylamide gel and electrophoresed until the XC tracking dye had migrated 17 cm into the gel. An autoradiograph of the gel was scanned with an Optronics drumscanner densitometer in conjunction with a VAX 11/780 computer. The resultant densitometer tracings, which plot optical density as a function of distance migrated into the gel, are shown here. Each peak is identified below by the length of its corresponding DNA fragment. The bottom trace corresponds to unreconstituted DNA fragments, the second trace from the bottom to mononucleosome reconstitutes, the third trace from the bottom to dinucleosomes, and the top trace to multinucleosomes.

of nonspecific pelleting of nucleoprotein complexes during sedimentation.

The identities of these peaks were confirmed by their relative positions in the gradient. The fractional distance moved by the dinucleosome peak, when divided by the distance moved by the mononucleosome peak, yields a ratio of 1.35. This number is in accord with the following ratios derived from previously published results: 1.30 (Rall et al., 1977), 1.33 (Kunkel & Martinson, 1981), and 1.35 (Chao, 1980). The position of the multinucleosome peak relative to that of the mononucleosome peak yields a ratio of 1.71. This number agrees with the ratio of 1.70 derived from the relative positions of trinucleosomes and mononucleosomes as published by Rall et al. (1977). Although these sucrose gradients are not strictly isokinetic, the close agreements between these ratios tend to confirm the identities of the peaks.

From Figure 1 it is apparent that although the majority of DNA fragments are shifted into the various nucleosome peaks after reconstitution (solid line), a small fraction of them do not associate with histones and thus remain near the top of the gradient. The fact that these unreconstituted fragments coincide mostly with the lightest portion of the free DNA marker peak (dashed line) indicates that the largest DNA fragment were probably driven preferentially into reconstitutes. The fragments that remain unreconstituted should therefore correspond to the fragments of shortest lengths.

In order to examine the actual distribution of DNA fragments across the sucrose gradient, each fraction collected from the gradient was run on an 8% polyacrylamide gel. The autoradiograph of such a gel is shown in Figure 2. The gel displayed here corresponds to the case where reconstitution was done at an intermediate histone-to-DNA ratio of 0.6. This gel shows that after reconstitution the DNA fragments are generally segregated along the sucrose gradient on the basis of length. Thus, the longest fragments are predominant in the lanes corresponding to multinucleosome fractions (lanes 1–3), fragments of intermediate length are predominant in nucleosome fractions (lanes 4–9), and the shorter fragments are predominant in the mononucleosome fractions (lanes 10–17). An exception to this trend is the 98-bp fragment (second from the bottom), which is found in all portions of the gradient. In addition, the 60-bp fragment (bottommost) does not appear in any fractions corresponding to reconstituted nucleosomes. Since the 60- and 98-bp fragments are smaller than nucleosome-length DNA and reconstitute in an anomalous fashion relative to the other fragments, they will not be considered in subsequent analyses of the data.

In order to facilitate the quantitative analysis of the behavior of each DNA fragment with respect to reconstitution, the protocol was modified as follows. First, appropriate sucrose gradient fractions were pooled and deproteinized prior to being loaded on a polyacrylamide gel. Each sucrose gradient corresponding to a separate reconstitution experiment was divided into four portions: unreconstituted DNA, mononucleosomes, dinucleosomes, and multinucleosomes. The fractions contained within each portion of the gradient were pooled separately and phenol-extracted prior to being applied to a single lane of a gel. Finally, the polyacrylamide gel was changed slightly to allow better resolution of the DNA fragments (see Materials and Methods).

Densitometer tracings of a typical autoradiograph resulting from this procedure are shown in Figure 3. The four superimposed scans correspond to four lanes of a single gel. The bottom scan corresponds to the pooled DNA fragments from the unreconstituted portion of the gradient (fractions 18–31). The second scan from the bottom corresponds to the pooled fragments from the mononucleosome portion of the gradient (fractions 11–17). The third and fourth scans from the bottom correspond to fragments from the dinucleosome (fractions 5–10) and multinucleosome peaks (fractions 1–4), respectively.

Each peak on the densitometer tracing shown in Figure 3 corresponds to a distinct DNA fragment, with the exception of the second peak from the left. This peak consists of both the 580- and 610-bp fragments, which could not be resolved on the gel. In all subsequent analyses we arbitrarily chose to treat this peak as being representative of the 580-bp fragment. The identities of the 237- and 240-bp fragments, as well as the 430- and 447-bp fragments, which are reversed in their expected order of migration, were determined by restriction enzyme analysis (see Materials and Methods).

Each DNA fragment in this assay was 5′ end-labeled with $^{32}PO_4$. The integrated area under each peak of the densitometer scan corresponds to the total radioactivity, and therefore the total molar amount of fragment present in a particular gel lane. This area can thus be used to calculate the proportion of fragment that is found in reconstituted vs. unreconstituted form. Since this analysis is dependent on the response of the film to the varying amounts of radioactivity present in the different DNA bands, only those integrated band intensities that fell within the linear range of the film response were used in this calculation (see Materials and Methods).

The data obtained from densitometer scans, such as those shown in Figure 3, can be used to calculate the extent to which each DNA fragment reconstitutes into mononucleosomes, dinucleosomes, or multinucleosomes. For example, the integrated intensity under the peak corresponding to the 286-bp fragment in the second trace from the bottom is 337.9 OD units. This represents the total amount of 286-bp fragment that reconstitutes as mononucleosomes. Dividing this value by the sum of the integrated intensities under the 286-bp fragment peak across all four lanes of the autoradiograph results in a figure of 337.9/1220.0, or 28%. This means that 28% of the fragment was reconstituted into mononucleosomes. Similarly, 50% of the 286-bp fragment reconstitutes as dinucleosomes, while only 14% of the fragment reconstitutes into more rapidly sedimenting structures.

Some fraction of the shortest fragments (172–286 bp) could be found in the multinucleosome peaks when reconstitutions were performed at relatively high histone-to-DNA ratios. This may imply that these fragments are capable of associating with multinucleosome complexes in which some of the histone octamers are not fully occupied by DNA of core particle length. The ability of histone octamers to bind fewer than 145 bp of DNA has been observed before (Klevan & Crothers, 1977; Tatchell & Van Holde, 1979). Since we only observed this phenomenon at high histone-to-DNA ratios, we do not think that it represents irreversible aggregation of certain DNA fragments with nucleoprotein complexes.

The partitioning of each fragment into mononucleosomes, dinucleosomes, and multinucleosomes changed with the length of the fragment and with the histone concentration used during reconstitution (data not shown). The example cited above illustrates the partitioning of a particular fragment at the highest histone-to-DNA (w/w) ratio of 1.0. At a ratio of 0.8, the 172- and 215-bp fragments reconstitute predominantly as mononucleosomes, those of length 237–430 bp reconstitutes predominantly as dinucleosomes, and those of 447–705 bp reconstitute predominantly as tri- or multinucleosomes. At an intermediate ratio of 0.6, fragments of length 172–286 bp reconstitute predominantly as mononucleosomes, those of 366–447 bp as dinucleosomes, and those of 580–705 bp as multinucleosomes. At the lowest histone-to-DNA ratio of 0.2, all of the fragments reconstitute predominantly as mononucleosomes. The latter observation will be an important feature of the experiments described below.

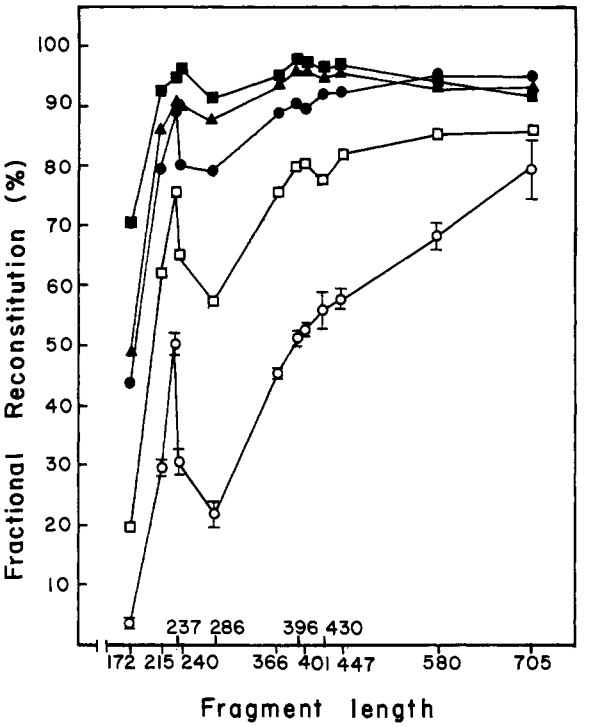

FIGURE 4: Fractional reconstitution vs. fragment length at varying histone-to-DNA ratios. SV40 DNA fragments were reconstituted with core histones at histone-to-DNA (w/w) ratios of 0.2 (○), 0.4 (□), 0.6 (●), 0.8 (▲), and 1.0 (■). Each sample was fractionated on sucrose gradients and then on polyacrylamide gels as described in the legend to Figure 3. Percent mononucleosome, percent dinucleosome, and percent multinucleosome was calculated for each fragment as described in the text. The fractional reconstitution (%) is the sum of percent mononucleosome, percent dinucleosome, and percent multinucleosome. The fragment lengths are plotted, from left to right, on a linear scale in terms of their relative lengths in base pairs. The errors in the measurement are shown only for the DNA fragments reconstituted at a histoner-to-DNA ratio of 0.2. This range of errors was representative of the errors associated with the other curves as well.

When reconstitution is performed at a histone-to-DNA ratio of 1.0, there is a sufficient concentration of histones available to ensure that, in most cases, at least 90% of the DNA fragments become associated with at least one nucleosome. If the concentration of histones is dropped relative to the concentration of DNA, however, the histones can become limiting during reconstitution. Under these conditions, differential abilities among the individual fragments to compete for histones should be observed experimentally.

In order to determine whether certain DNA fragments actually have different affinities for histones, we reconstituted the mixture of SV40 restriction fragments using five different histone-to-DNA ratios, ranging from 0.2 to 1.0. In Figure 4 these results are plotted as the fraction of fragment reconstituted at each of the five histone-to-DNA ratios vs. fragment length. At the highest histone-to-DNA ratio of 1.0 (filled squares), at least 90% of each fragment reconstitutes into mononucleosomes or higher order complexes, with the exception of the shortest fragment. As the histone-to-DNA ratio is decreased, however, the longer fragments appear to remain more reconstituted relative to the shorter fragments. This differential effect of DNA length on the extent of reconstitution has also been observed for a mixture of pBR322 restriction fragments (Linxweiler & Hörz, 1984). At the lowest histone-to-DNA ratio of 0.2 (open circles), the reconstituted fraction of the 215, 240, and 366-bp fragments and larger fragments follows a smooth curve with increasing fragment

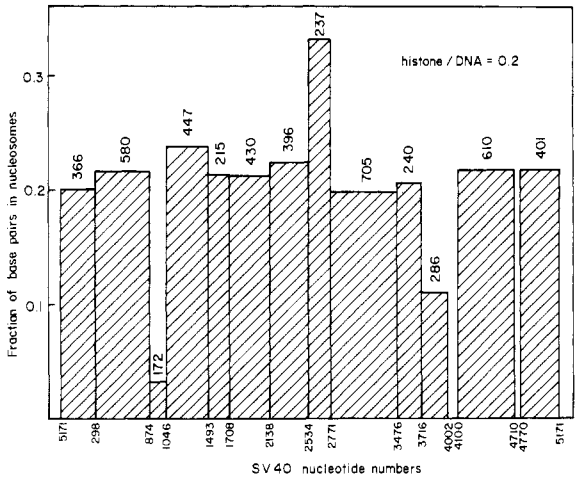

FIGURE 5: Fraction of base pairs in nucleosomes displayed vs. a linear representation of SV40 genome. The fractional reconstitution of each fragment at a histone-to-DNA ratio of 0.2 (from Figure 4) was used to calculate the fraction of base pairs in nucleosomes by the formula given in text. This value is plotted against the position of each DNA fragment in the SV40 genome. The length of each fragment in base pairs is indicated above each bar. Fragment boundaries are reported below the graph in terms of SV40 nucleotide numbers (Tooze, 1981). The gaps between nucleotides 4002–4100 and 4710–4770 correspond to the positions of the 98- and 60-bp fragments, which were not analyzed (see Results). The fraction of mononucleosomes formed by each fragment was as follows: 705 (64%), 610/580 (51%), 447 (43%), 430 (49%), 401 (45%), 396 (41%), 366 (41%), 286 (20%), 240 (28%), 237 (46%), 215 (27%), and 172 (3.8%).

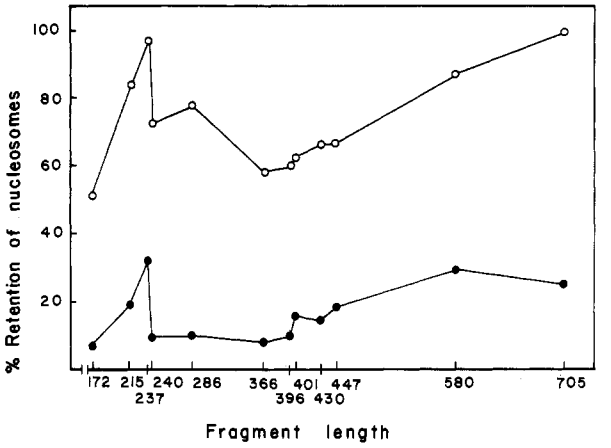

FIGURE 6: Effect of heparin on SV40 reconstitutes. SV40 DNA fragments were reconstituted with core histones at a histone-to-DNA ratio of 1.0. After reconstitution was complete, heparin was added to a final concentration of either 0.226 (○) of 1.13 mg/mL (●), and the samples were incubated at 37 °C for an additional hour. Samples were fractionated on 5–20% sucrose gradients and then on polyacrylamide gels, along with controls that had not been treated with heparin. Fractional reconstitution was calculated for each fragment as described in the legend to Figure 4. The total number of nucleosomes associated with each fragment was calculated as described in the text, and the retention of nucleosomes after heparin challenge was expressed as a percentage of the number remaining relative to the untreated control. This percent retention of nucleosomes is plotted against fragment length.

length. However, some of the shorter fragments, in particular the 172, 237, and 286-bp fragments, clearly deviate from this curve, and this tendency is evident at the other low histone-to-DNA ratios as well.

In order to examine the intrinsic ability of the various fragments to compete for the available histones, we chose the lowest histone-to-DNA ratio of 0.2. At this ratio, where all of the fragments reconstitute predominantly as mononucleosomes, individual differences in the fragments' abilities to reconstitute are most apparent. However, since longer fragments are likely to possess more potential histone binding sites, they should be able to compete more readily for histones than shorter fragments. In order to correct for the advantage of longer DNA in competing for histones, we calculated the fraction of the total number of base pairs in a given fragment that was occupied by nucleosomes. If we assume that each nucleosome covers 145 bp of DNA, then the fraction of base pairs in nucleosomes is 145 bp × [1(% mononucleosome) + 2(% dinucleosome) + 3(% multinucleosome)]/[100 × fragment length (bp)]. Under conditions of limiting histone concentration, where dinucleosomes and higher order complexes are not formed to any significant extent, the fraction of base pairs occupied by nucleosomes should reflect the ability of each fragment to form reconstitutes, regardless of fragment length. In principle, if all of the sequences represented on these fragments compete equally well for histones, then all of the fragments should possess the same value for fraction of base pairs in nucleosomes.

Figure 5 depictes the derived values for fraction of base pairs in nucleosomes for each fragment at a histone-to-DNA ratio of 0.2. These are plotted vs. their order along the SV40 genome. When the data are displayed in this fashion, it is evident that for most of the fragments the above expectation is met; they compete to very similar extents in forming mononucleosomes. One exception is the 237-bp fragment, which forms nucleosomes exceptionally well compared to the majority of SV40 fragments. Two other fragments, the 172- and 286-bp fragments, compete relatively poorly for histones. These results are in general agreement with previous studies showing that various regions of intact SV40 DNA reconstitute with different preferences in vitro (Wasylyk et al., 1979; Simpson & Stein, 1980; Hiwasa et al., 1981). Like Simpson and Stein (1980), we also see no evidence that sequences near the origin of replication (i.e., the 366-bp fragment) reconstitute less readily than the remainder of the genome.

Since our results could imply that certain fragments may form nucleosome reconstitutes of significantly altered stability, we decided to test this possibility directly by challenging the reconstituted SV40 fragments with the polyanion heparin, which is known to destabilize DNA–histone interactions (Ansevin et al., 1975). The resistance of the nucleosome complexes to disruption could then be measured by estimating the number of nucleosomes that were lost from each DNA fragment as a result of the exposure to heparin.

Figure 6 displays the percent retention of nucleosomes following heparin challenge for each of the SV40 fragments. The retention of nucleosomes is expressed as the percentage of nucleosomes remaining after heparin challenge relative to the unchallenged control. The total number of nucleosomes associated with a particular fragment was calculated as being proportional to 3 × percent multinucleosome plus 2 × percent dinucleosome plus 1 × percent mononucleosome. This approximation may underestimate the number of nucleosomes associated with the longest fragments since the multinucleosome peaks are likely to contain complexes of a higher order than trinucleosomes. However, since a calculation of nucleosome retention based solely on the change in the total fraction of fragment remaining reconstituted after heparin challenge would not account for the loss of one nucleosome from a trinucleosome to a dinucleosome, or a dinucleosome to a mononucleosome, we feel that this analysis provides a valid measure of overall nucleosome stability.

The lowest heparin concentration used, 0.226 mg/mL, represents a 2-fold weight excess of heparin to total DNA. Under these conditions, certain fragments form reconstitutes

that are obviously more stable than others since they retain virtually all of their nucleosomes upon challenge (Figure 6, open circles). This apparent resistance to disruption is likely to be an artifact for the two largest fragments, since the loss of nucleosomes from multinucleosome complexes would be underestimated by this analysis. However, the 215-bp and especially the 237-bp reconstitutes, which are also relatively stable to heparin challenge, should not be subject to this artifact.

In order to corroborate the observed variations in nucleosome retention, the nucleosome population was challenged with 1.13 mg/mL heparin. Under these conditions, which represent a 10-fold weight excess of heparin to DNA, the larger fragments lose a sufficient number of nucleosomes that any observed stability of the larger reconstitutes should be less subject to artifact. The apparent enhanced stability of the larger reconstitutes relative to the shorter reconstitutes is greatly diminished, but it is obvious that, even under these stringent conditions of challenge, the 237-bp reconstitute remains markedly stable (Figure 6, closed circles).

Discussion

Reconstitution Potential Is Affected by DNA Sequence. Our results demonstrate that at least three restriction fragments, containing widely separated portions of the SV40 genome, reconstitute into nucleosome at levels that differ markedly from those of the remainder of the SV40 fragments. In order to discriminate between the abilities of the various fragments to reconstitute, the weight ratio of histone to DNA was varied over a 5-fold range during the reconstitution, from 1.0 to 0.2. Reconstitution at a histone-to-DNA ratio of about 0.8 corresponds to conditions that should give rise to approximately the same complement of core histones as would be found complexed with SV40 DNA in vivo. Consequently, a histone-to-DNA ratio of 0.2 should result in reconstitution of only one-fourth this number of nucleosomes. When the fractional reconstitution of each DNA fragment at a histone-to-DNA ratio of 0.2 is converted to fraction of base pairs in nucleosomes, 10 of the 13 fragments reconstitute with similar abilities. The average fraction of base pairs that are found in nucleosomes for these 10 fragments is 0.21 (see Figure 5). The three remaining fragments reconstitute in an anomalous manner. Two fragments are underreconstituted relative to the norm. The 172-bp fragment, which spans nucleotides 874–1046, reconstitutes at a level that is only 0.032/0.21 or 0.15 times the average value for all of the fragments considered together. The 286-bp fragment, which spans nucleotides 3716–4002, reconstitutes at only 0.11/0.21 or 0.53 times the average. In contrast to the inability of the 172- and 286-bp fragments to reconstitute as well as the norm, one fragment reconstitutes substantially better than the others. This fragment, which is 237 bp in length and spans nucleotides 2534–2770, reconstitutes 0.33/0.21 or 1.6 times more efficiently than the norm. These numerical estimates of the relative abilities of these fragments to reconstitute apply to one chosen set of experimental conditions and will change with higher or lower histone-to-DNA ratios. Given these conditions, however, there is an order of magnitude variation in the ability of these fragments to reconstitute, such that the binding of histones to the 237-bp fragment is favored 10-fold over binding to the 172-bp fragment.

Another observation that can be derived from the data illustrated in Figures 4 and 5 is that the 366-bp fragment, which spans nucleotides 5172–298, reconstitutes in a normal manner compared to the majority of fragments representing the SV40 genome. In addition, the 366-bp fragment does not appear to be abnormally stable or unstable to heparin challenge. These observations are of interest since the 366-bp fragment contians the origin of replication, the 72-bp repeats, and several of the promoters for both early and late mRNA transcription. Since these sequences coincide with the region of DNA that was reported to be nucleosome free in a subpopulation of SV40 minichromosomes examined during lytic infection, it is reasonable to conclude that, on the basis our results, the lack of nucleosomes observed on these sequences in vivo does not arise from an inability of these sequences to support stable nucleosome formation; rather, this effect must be due to some other property of SV40 chromatin.

The 237-bp Fragment Forms a Hyperstable Reconstitute. As another measure of the affinity of the SV40 DNA sequences for histones, we examined the stability of the reconstituted nucleosomes upon exposure to the polyanion heparin. As expected, incubation of nucleosomes with heparin resulted in the loss of histones from SV40 fragments. The shortest DNA fragments exhibit a great deal of variation in their ability to retain histone cores, likely due to their differing DNA sequences. The longer fragments appear to form more resistant reconstitutes than the shorter fragments; however, this is largely a property of their length rather than sequence, since our analysis does not score for the loss of nucleosomes from multinucleosomes to trinucleosomes (see Results).

We suspect that this challenge assay does not measure the true thermodynamic stability of a nucleosome complex. Rather, it may test the complex's resistance to disruption by a destabilizing agent. This assay may thus indicate the ability of a nucleosome to resist being removed from DNA, a parameter that may be important in biological processes such as transcription or replication.

The largest stability difference is exhibited by the 237-bp reconstitute, whose nucleosomes are seen to be much more stable to heparin challenge when compared to the others. In fact, under the conditions of lowest heparin challenge, a calculation shows that the nucleosomes associated with the 237-bp fragment are roughly 10 times more stable than the median, since only 3% of the histone cores are lost compared to the median loss of about 30%. The enhanced stability of the 237-bp reconstitute is a novel observation that appears to be a unique property of the DNA sequences found on this fragment.

Several peculiarities exist in the sequence of the 237-bp fragment, including a stretch of seven dA residues located between nucleotides 2593 and 2599, as well as a 12-bp direct repeat (with a 17-bp spacer), beginning at nucleotide 2600. These features could directly contribute to the enhanced ability of this fragment to form stable nucleosomal structures. Also, as noted under Materials and Methods, the 237-bp fragment migrates in an anomalous manner in polyacrylamide gels. This behavior, which has been correlated with the ability to assume abnormal DNA structures, such as bent DNA, could presumably bear upon the enhanced ability of this fragment to reconstitute into stable nucleosomes (Marini et al., 1982; Drew & Travers, 1985). Studies are currently under way to investigate the basis for the apparent gel retardation of the 237-bp fragment. In contrast to the 237-bp fragment, the 172- and 286-bp fragments, which reconstitute inefficiently into nucleosomes do not possess any obvious abnormalities in their DNA sequences. The basis for their relative inabilities to reconstitute is therefore unclear.

Our results indicate that three fragments reconstitute with different efficiencies relative to the majority of fragments. The 237-bp fragment, which reconstitutes 1.6 times more efficiently

than the norm, has a length that falls in between the 172- and 286-bp fragments, both of which reconstitute much less efficiently than the norm (0.15- and 0.53-fold, respectively). This observation argues against the differential reconstitution abilities of these fragments being primarily a function of length and not of sequence. If this were the case, the 237-bp fragment should reconstitute less well than the 286-bp fragment, on the basis of its shorter length. In addition, the enhanced ability of the 237-bp fragment to reconstitute cannot be a property of its length alone since it differs by only 3 bp from the 240-bp fragment, which reconstitutes in a normal fashion. Since the 172-bp fragment is one of the shortest in this mixture, its instability could in principle be due to an end effect whereby the ends of the fragment could position the DNA asymmetrically on the protein core (Tatchell & Van Holde, 1979). However, this possibility seems unlikely since Tatchell and Van Holde (1979) did not observe such an end effect with nucleosomes reconstituted from DNA fragments of 177 bp in length. In addition, the length of this fragment coincides closely with the observed length (approximately 168 bp) of chromatosome particle DNA isolated from micrococcal nuclease digestions of whole chromatin in vivo [reviewed in McGhee and Felsenfeld (1980)]. Thus, the 172-bp fragment should contain more than an adequate number of binding sites to enable it to fully contact a histone octamer.

A consideration in the interpretation of these results is that in longer fragments the potential effects of shorter sequence elements may be masked. Thus a short sequence that was markedly inefficient at binding histones might not influence the overall reconstitution ability of a long fragment such as the 705-bp fragment, especially if reconstitution was performed with limiting histone concentrations. Under these conditions, only the most favorable set of DNA–histone interactions would be selected from the mixture of possible interactions available on a given fragment. Therefore, it is probable that the fragments that we singled out for their reduced abilities to reconstitute (the 172- and 286-bp fragments) comprise only a subset of the DNA sequences that could potentially exert such an effect. On the other hand, an increased ability to reconstitute, as seen in the 237-bp fragment, should still be detected by our method of analysis.

Another factor that could influence our ability to detect sequence-specific reconstitution preferences is the choice of reconstitution protocol. Our reconstitution procedure may be only quasi-thermodynamic in nature, with some contribution from irreversible kinetic processes. If irreversible histone–DNA associations can occur during reconstitution, then potential reconstitution preferences among the different sequences may be masked or partially obscured. It is thus conceivable that more diversity would be observed among the reconstitution preferences of these sequences if the experiments were to be repeated with a reconstitution procedure that more closely approaches a state of equilibrium thermodynamics.

A Possible Role for Hyperstable Nucleosome in SV40 Termination Events. DNA sequences that can either readily form nucleosomes or discourage their formation may play an important role in the maintenance of a specific chromatin structure in vivo. We therefore examined the potential role of the 172-, 237-, and 286-bp fragments in terms of the relationship between their abilities to reconstitute and their position on the SV40 genome. The 172-bp fragment, which includes nucleotides 874–1046, lies within the sequences that encode the late viral messenger RNA. The 286-bp fragment, which includes nucleotides 3716–4002, lies within the sequences coding for early viral messenger RNA. Both of these fragments are inefficient at reconstitution relative to the remainder of the genome. However, since both of these sequences are located in the middle of long portions of transcribed DNA, it is difficult to imagine what specific role they could play in vivo. On the other hand, the 237-bp fragment (nucleotides 2534–2770), which is more efficient at reconstitution and which forms more stable nucleosomes than the average SV40 sequence, lies in a potentially more interesting part of the genome.

DNA replication normally terminates at around 0.17 map unit [reviewed in Tooze (1981)], in a region that is contained within the 237-bp fragment. It is known that unique DNA sequences are not required for this process, since the analysis of evolutionary variants has shown that termination preferentially occurs at sites that are located approximately 180° from the site of initiation (Brockman et al., 1975). Nevertheless, these results do not rule out the possibility that termination may occur more efficiently at certain preferred DNA sequences. In fact, preferred sites for pausing of the replication fork have been observed in replicating SV40 (Tapper & DePamphilis, 1980; Zannis-Hadjopoulos et al., 1983), and it has been proposed that these could arise from blockage of the replication fork by specifically placed nucleosomes (Trifonov & Mengeritsky, 1984). In addition, sequences in the termination region may affect the ease with which SV40 replication intermediates are unwound and decatenated (Weaver et al., 1985). Thus, it is possible that the existence of a markedly stable nucleosome could assist the termination of replication by minimizing read-through by the replication complex into sequences less efficient at termination and possibly, decatenation. It is also conceivable that a specifically placed nucleosome could assist in the complicated process of DNA strand separation after replication is completed. In fact, recent evidence suggests that nucleosomes may indeed be present in the termination region of late replicating SV40 intermediates (Sogo et al., 1986).

The 3′ termini for early mRNA have been mapped to nucleotides 2586 and 2587 (Lebowitz & Weissman, 1979). Although it is not clear whether these termini arise from termination of transcription or from processing of precursor RNA molecules, it has been postulated that transcription might terminate near a stretch of seven dA residues at nucleotides 2593–2599 [see Lebowitz and Weissman (1979)]. If this is true, it is easy to imagine how a stable nucleosome complex in this region might enhance termination of transcription by either physically blocking a transcription complex or by exposing DNA sequences serving as termination signals in an appropriate conformation.

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Competitive Labeling as an Approach to Defining the Binding Surfaces of Proteins: Binding of Monomeric Insulin to Lipid Bilayers†

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ABSTRACT: The free monomeric form of insulin is known to adsorb strongly to many different surfaces. A question of physiological relevance for which no previous studies have been reported is whether the monomeric form of insulin binds to lipid bilayers. In order to answer this question, it is necessary to carry out studies at the very dilute concentrations (less than 10^{-6} M) necessary to obtain this species. We have approached this problem by applying the method of competitive labeling [Hefford, M. A., Evans, R. M., Oda, G., & Kaplan, H. (1985) *Biochemistry 24*, 867-874] to study insulin at concentrations as low as 3×10^{-8} M, in the presence and absence of large unilammelar liposomes. With 1-fluoro-2,4-dinitrobenzene as the labeling reagent, the relative chemical reactivities of the functional groups of insulin were found to decrease markedly when insulin was incubated with liposomes consisting of egg lecithin and cholesterol (2:1 mol/mol) in 1.0 M KCl, pH 7.5 at 37 °C. The decrease for each functional group was found to directly correlate with its proximity to the dimer-forming surface of the monomer. It is concluded that insulin binds to lipid bilayers in a specific orientation, with the dimer-forming surface interacting with the bilayer. These results demonstrate the feasibility of applying competitive labeling to obtain structure–function relationships of membrane-interactive proteins in general and monomeric insulin in particular.

Insulin, at the very dilute physiological concentrations where the free monomer is the predominant species, is widely known to strongly adsorb[1] to many different surfaces; in spite of this, there have been very few quantitative studies on this phenomenon. Hollenberg and Cuatrecasas (1976) reported an

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[1] Adsorption refers to the association between two components where the orientation is random or not defined. Binding refers to an association between two components where the orientation is known to be nonrandom.

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